JOURNAL OF BACTERIOLOGY, Apr. 1983, p. 1-9
0021-9193/83/040001-09$02.00/0

Vol. 154, No. 1

Plasmid Complements of *Streptococcus lactis* NCDO 712 and Other Lactic Streptococci After Protoplast-Induced Curing

MICHAEL J. GASSON

National Institute for Research in Dairying, Shinfield, Reading, Berkshire RG2 9AT, England

Received 13 July 1982/Accepted 15 November 1982

The production and regeneration of bacterial protoplasts promoted the loss of three different plasmid-specified traits in *Streptococcus lactis* subsp. *diacetylactis* strains. The loss of five different plasmids, including small multicopy molecules, was readily detected in *Streptococcus lactis* 712 by screening lysates of random protoplast regenerants on agarose gels. In this strain sequential rounds of protoplast regeneration were used to produce a plasmid-free strain and derivatives carrying only single molecules from the plasmid complement. During these experiments a 33-megadalton plasmid, pLP712, was found to encode genes for lactose and protein utilization. Only this plasmid was required for normal growth and acid production in milk; the remaining four plasmids appeared to be cryptic. Lactose-defective derivatives of a strain carrying only pLP712 were readily isolated. Although these derivatives included instances of plasmid loss, deletions of pLP712 were frequently found. Many different deleted derivatives of pLP712, including some in which the lactose or protein utilization determinant or both were lost, were isolated. The molecular instability of pLP712 largely accounted for previous observations of plasmid complements in *S. lactis* 712 after lactose determinant curing or transfer by conjugation and transduction. Curing of cryptic molecules from multiple plasmid complements by protoplast regeneration may prove to be generally valuable in lactic streptococci and other gram-positive species.

Most strains of *Streptococcus cremoris*, *Streptococcus lactis*, and *Streptococcus lactis* subsp. *diacetylactis* carry a large complement of plasmids (1, 2, 15, 16, 21). This characteristic can pose problems in the assignment of suspected plasmid-encoded phenotypes to individual molecules. It also complicates the analysis of plasmid transfer experiments (1, 7) and the molecular study of individual plasmids from the complement (M. J. Gasson, P. J. Warner, and J. Elkins, Abstr. Soc. Gen. Microbiol. 94th, Cambridge, England, abstr. no. M8, 1982): In *S. lactis* 712 the ability to utilize lactose was lost at a high frequency, which was increased by standard plasmid curing treatments. The lactose-negative variants so produced were nonrevertable, and the lactose utilization phenotype could be transferred by conjugation at frequencies as high as 0.6 per recipient colony (7). Despite the strong suggestion from these genetic properties that lactose utilization was plasmid encoded, physical identification of a lactose plasmid in *S. lactis* 712 was not achieved (1, 7). Although some lactose-negative variants had lost various plasmid molecules, no consistent correlation between these changes was found. Furthermore, other lactose-negative variants were isolated that had apparently unaltered plasmid profiles (1, 7). Analysis of the plasmid complements of *S. lactis* 712 strains in which lactose genes had been transferred either by conjugation or by transduction revealed complex changes, which included the appearance of various novel plasmid molecules (1, 5). In gene transfer and plasmid curing experiments, genetic linkage was observed between the ability to utilize lactose and milk protein (1, 5, 7). Because this association was not always maintained (1, 5, 7), a further complication concerned the nature of the molecular relationship between these two apparently plasmid-linked genotypes in *S. lactis* 712 (1).

In *Staphylococcus aureus* (20), *Streptomyces* species (10), and *Bacillus subtilis* (3), plasmid curing after the regeneration of bacterial protoplasts has been reported. The development of a protocol for protoplast production and regeneration in lactic streptococci (4) allowed this phenomenon to be investigated in *Streptococcus* species. Here, protoplast-promoted curing was shown for a variety of plasmid phenotypes in *S. lactis* subsp. *diacetylactis* strains and exploited

TABLE 1. Bacterial strains

Strain no.	Derivation[a]	Reference
MG317	*S. lactis* subsp. *diacetylactis* NCDO 176(pAMβ)	6
MG318	*S. lactis* subsp. *diacetylactis* NCDO 823(pAMβ)	6
MG320	*S. lactis* subsp. *diacetylactis* NCDO 1008(pAMβ)	6
SH4109[b]	*S. lactis* NCDO 712 cured of φT712 prophage	8
MG1363	Plasmid-free derivative of SH4109	This paper
MG1614	Str[r] rif[r] derivative of MG1363	This paper
MG1299	Plasmid-cured SH4109 carrying only pLP712	This paper
MG1365	Plasmid-cured SH4109 carrying only pSH71	This paper
MG1362	Plasmid-cured SH4109 carrying only pSH72	This paper
MG1261	Plasmid-cured SH4109 carrying only pSH73	This paper

[a] NCDO cultures were from the National Collection of Dairy Organisms, National Institute for Research in Dairying, Shinfield, Reading, England. Str[r] and Rif[r] indicate resistance to streptomycin (200 μg/ml) and rifampin (100 μg/ml), respectively.

[b] The plasmid complement of SH4109 consists of pLP712, pSH74, pSH73, pSH72, and pSH71, which are, respectively, a 33-Md lactose and protein utilization plasmid and cryptic molecules of 9, 5.2, 2.5, and 1.8 Md.

in *S. lactis* 712 to produce a plasmid-free strain and derivatives carrying only single plasmids. During these experiments a 33-megadalton Md plasmid encoding genes for lactose utilization and protease production was identified. This plasmid showed an unusual degree of molecular instability, a characteristic that largely accounted for the observed complexity of the *S. lactis* 712 plasmid complement previously reported (1, 5, 7).

(A preliminary report of this work was presented to the International Conference on Streptococcal Genetics sponsored by the American Society for Microbiology, 9 to 12 November 1981, Sarasota, Fla. [6].)

MATERIALS AND METHODS

Bacterial strains. The strains used are described in Tables 1 and 2.

Media and reagents. Most of the media and reagents used were described by Gasson and Davies (7) and Gasson (4). The improved medium for detection of citrate fermenting ability developed by Kempler and McKay (13) was used. The restriction endonucleases *Bcl*I, *Hae*II, and *Hin*dIII were purchased from Bethesda Research Laboratories, Inc., Bethesda, Md., and *Eco*RI was from Boehringer Mannheim Corp., New York, N.Y. Storage buffers and reaction buffers were used according to the manufacturers' recommendations. Cesium chloride, AnalaR grade, was purchased from BDH, Poole, England.

Detection of metabolic phenotypes. The ability of strains to utilize milk protein, lactose, and citrate was determined by stab testing into appropriately supplemented citrated milk agar (7, 22), bromocresol purple lactose indicator agar (7, 19), and improved citrate detection medium (13), respectively.

Plasmid curing. The protocols for acriflavine and heat curing of plasmids were described by Gasson and Davies (7).

Plasmid transfer. The procedures for lactose plasmid transfer by conjugation and differentiation of Lax^- aggregating from Lax^+ nonaggregating donors were described by Gasson and Davies (7). For transduction, 0.1 ml of an 18-h culture of recipient bacteria, 0.1 ml of phage lysate, and 0.05 ml of 1 M $CaCl_2 \cdot 2H_2O$ were mixed and incubated at room temperature for 10 min. Transductants were selected by plating on lactose M17 agar and incubation for 48 h at 30°C. Transduction frequencies were expressed as transductants per PFU. UV light induction of phage and plaque assays were done as described by Gasson and Davies (8).

Protoplasts. The techniques for the preparation, fusion, and regeneration of lactic streptococcal protoplasts were described by Gasson (4).

Growth and acid production in milk. Overnight cultures grown in M17 broth were washed in sterile distilled water, and a 1% inoculum was made into skimmed milk. Samples were taken immediately and at 1-h intervals during incubation at 30°C. Cell numbers were determined by plating dilutions on M17 agar, and acidity was determined by neutralization with 0.1 M NaOH.

Routine plasmid extraction. Extraction of extrachromosomal DNA to examine the plasmid complements of selected strains was done by the protocol described by Gasson and Davies (7).

Rapid plasmid screening. A new procedure was developed for rapid screening of the plasmid complements from large numbers of protoplast regenerants. A 10% inoculum from an overnight culture was made in

TABLE 2. Plasmid curing after regeneration of *S. lactis* subsp. *diacetylactis* protoplasts

Strain no.	Prepn[a]	% Loss of phenotype[b]		
		Lac	Ery[r]	Cit
MG317	Control cells	<1	<1	<1
	Protoplast regenerants	<1	1	1
	PEG-treated protoplast regenerants	2	3	2
MG318	Control cells	<1	<1	<1
	Protoplast regenerants	15	<1	11
	PEG-treated protoplast regenerants	8	1	7
MG320	Control cells	<1	<1	<1
	Protoplast regenerants	22	1	4
	PEG-treated protoplast regenerants	13	1	1

[a] PEG, Polyethylene glycol.

[b] Ery[r], Erythromycin resistance.

10 ml of M17 medium and incubated for 2 h at 30°C. Cells were pelleted by centrifugation, and the supernatant fluids were thoroughly drained off. Cells were suspended in 250 μl of a 4-mg/ml lysozyme solution in protoplast buffer (4) and incubated at 37°C for 5 min. A 12.5-μl amount of diethyl pyrocarbonate was added, and incubation was continued for a further 10 min. The cells were lysed by adding 250 μl of final lysis mix (7), and 125 μl of 5 M sodium chloride solution was added. After 30 min of storage on ice, lysates were cleared by centrifugation for 30 min at 15,000 rpm in a Beckman JA20.1 rotor and Beckman J2-21 centrifuge. Decanted supernatant fluids were deproteinized with chloroform-isoamyl alcohol (7), and DNA was precipitated overnight at −20°C with ethanol (7). Nucleic acid was harvested by centrifugation at 10,000 rpm in a Beckman JA 20.1 rotor, ethanol was removed, and the pellets were suspended in 20 μl of TE buffer. RNA was sometimes removed by digestion with RNase (7).

Large-scale isolation of plasmid DNA. Lysis was performed with 500-ml batches of cells freshly grown to late exponential phase (optical density at 600 nm, 0.6 to 0.8). Cells were harvested by centrifugation, washed in sterile distilled water, suspended in 12.5 ml of a 4-mg/ml solution of lysozyme in protoplast buffer (4), and then transferred into 50-ml Oak Ridge centrifuge tubes. After 5 min of incubation at 37°C, 250 μl of diethyl pyrocarbonate was added, and incubation was continued for another 10 min. Final lysis mix (12.5 ml) was added, followed by 6.25 ml of 5 M sodium chloride. Lysates were held on ice for 30 min and cleared by centrifugation for 30 min at 15,000 rpm in a Beckman JA17 rotor and Beckman J2-21 centrifuge. The supernatant fluids were decanted into fresh centrifuge tubes and deproteinized by extraction with chloroform-isoamyl alcohol (7). Ethanol-precipitated nucleic acid was harvested by centrifugation, and dried pellets were dissolved in TE buffer (7) and digested with RNase (7). DNA extracted from 1.5 liters of cells was made up to a 13-ml volume in TE buffer containing 10.4 g of cesium chloride, and the refractive index was adjusted to 1.395. Cesium chloride and DNA, together with 0.2 ml of 10-mg/ml ethidium bromide, were filled into 13-ml Beckman Quickseal tubes (Beckman catalog no. 342413) and centrifuged at 40,000 rpm for 44 to 65 h at 10°C in a 75 Ti Beckman rotor and Beckman L5-65 ultracentrifuge. Covalently closed circular DNA was removed from the gradient by horizontal insertion of a syringe and extracted three to four times with equilibrated isoamyl alcohol to remove the ethidium bromide. The DNA was extensively dialyzed against TE buffer, ethanol precipitated, suspended in 50 to 100 μl of TE buffer, and stored at 4°C in 400-μl Eppendorf tubes.

Restriction enzyme digestion. The DNA concentration was adjusted to give good resolution of restriction endonuclease digests from 2 μl of solution. Digests were performed for 2 to 4 h in the enzyme manufacturer's recommended reaction buffer, using 2 μl of diluted enzyme and 2 μl of the DNA solution in a final volume of 20 μl.

Agarose gel electrophoresis. The procedure for agarose gel electrophoresis were described by Gasson and Davies (7). Vertical gel apparati were used for restriction endonuclease digests and routine plasmid complement analysis. Rapid screening of plasmid complements was performed on horizontal gels.

RESULTS

Plasmid curing after protoplast production and regeneration in *S. lactis* subsp. *diacetylactis*. Three strains with different plasmid complements were used. The erythromycin resistance plasmid pAMβ was introduced into each strain by conjugation from *Streptococcus faecalis* DS-5, with *S. lactis* 712 used as an intermediate donor (6) (strains MG317, MG318, and MG320). In addition to the erythromycin resistance marker of pAMβ, curing of the apparently plasmid-linked lactose and citrate utilization phenotypes (11, 12, 14) was investigated. Random protoplast regenerants and control bacteria were patched onto glucose-M17 agar. Patches were replica plated onto glucose-M17 agar containing erythromycin to score loss of pAMβ. Curing of the lactose and citrate utilization phenotypes was checked by stab testing the same patches into lactose-bromocresol purple agar and McKay citrate agar, respectively. Curing of all three plasmid-associated traits was detected after protoplast regeneration (Table 2), but was not found in control cells. In addition, plasmid curing after treatment with the protoplast fusion agent polyethylene glycol was investigated (Table 2). In general, this agent neither increased nor decreased the frequency of plasmid loss.

Representative cured derivatives were checked by agarose gel electrophoresis of cleared lysates. In erythromycin-sensitive derivatives of strains MG317, MG318, and MG320, the 17-Md pAMβ plasmid was lost; similarly, the citrate-negative derivatives of all three strains were cured of a 5.5-Md plasmid. Lactose-defective derivatives of *S. lactis* subsp. *diacetylactis* MG320 lost a 35-Md molecule. In contrast, lactose-defective derivatives of strains MG317 and MG318 showed no change in the plasmid profile. In addition, some protoplast regenerants of strain MG318 had lost an apparently cryptic 1-Md plasmid without a correlating change of phenotype. An agarose gel showing the plasmid curing in *S. lactis* subsp. *diacetylactis* MG320 is shown in Fig. 1.

The investigation of curing in *Staphylococcus aureus* protoplasts led to the hypothesis that plasmid loss occurs during the early stages of protoplast regeneration (20). This conclusion was based on the observation that regenerant colonies often consisted of a mixture of plasmid-cured and plasmid-carrying cells. In *S. lactis* subsp. *diacetylactis* cells a similar phenomenon was frequently encountered. For instance, in an experiment with *S. lactis* subsp. *diacetylactis* 176, protoplast-regenerant colonies were segregated by streaking onto glucose-M17 agar. Ten single colonies from each regenerant were then tested for their ability to utilize citrate. Of the 20 regenerant colonies investigated, 14 were >90%

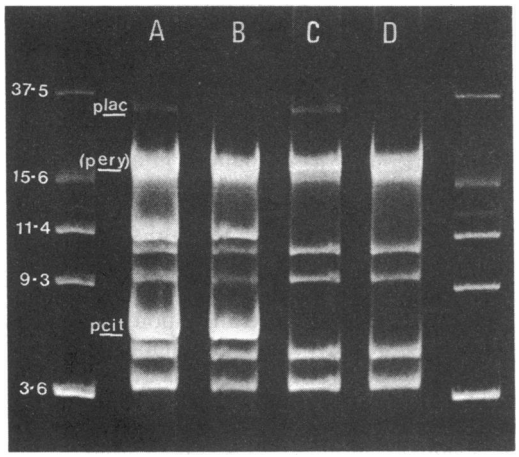

FIG. 1. Plasmid curing after regeneration of protoplasts from *S. lactis* subsp. *diacetylactis* MG320. Plasmid profiles are shown for regenerants with (A) unchanged Lac^+ Cit^+, (B) Lac^- Cit^+, (C) Lac^+ Cit^-, and (D) Lac^- Cit^- phenotypes. The erythromycin resistance plasmid pAMβ (pery) migrated with chromosomal DNA.

citrate positive, 2 were >90% citrate negative, and 4 were mixtures of positive and negative cells.

Plasmid curing after protoplast production and regeneration in *S. lactis* 712. *S. lactis* 712 carried five plasmids, with molecular sizes of 33, 9, 5.2, 2.5, and 1.8 Md. The lactose and protein utilization phenotypes were frequently lost after protoplast regeneration, and curing of the apparently cryptic plasmids could be readily detected by screening random regenerants in a rapid lysis and agarose gel procedure. Sequential rounds of protoplast production and regeneration were used to produce a plasmid-free strain and strains carrying only a single plasmid. In one series of experiments the lactose and protein utilization phenotypes were checked after the plasmid profiles were examined, whereas in a second series only the regenerants that retained these phenotypes were tested for plasmid loss (Table 2 and Fig. 2). A plasmid-free strain and derivatives carrying only a single plasmid were isolated with relatively little difficulty. The complete series of derivatives (Fig. 2), except for one carrying the exceptionally unstable 9-Md plasmid, were obtained from 210 protoplast regenerants. Derivatives that had lost the 33-Md plasmid were always lactose defective, whereas those strains retaining a molecule of this size could be either lactose positive or lactose negative (Table 2). Sequential rounds of plasmid curing from lactose-positive protoplast regenerants eventually led to the isolation of a strain, MG1299, which retained only one 33-Md molecule (Fig. 2H). The ability of this strain to grow in milk was

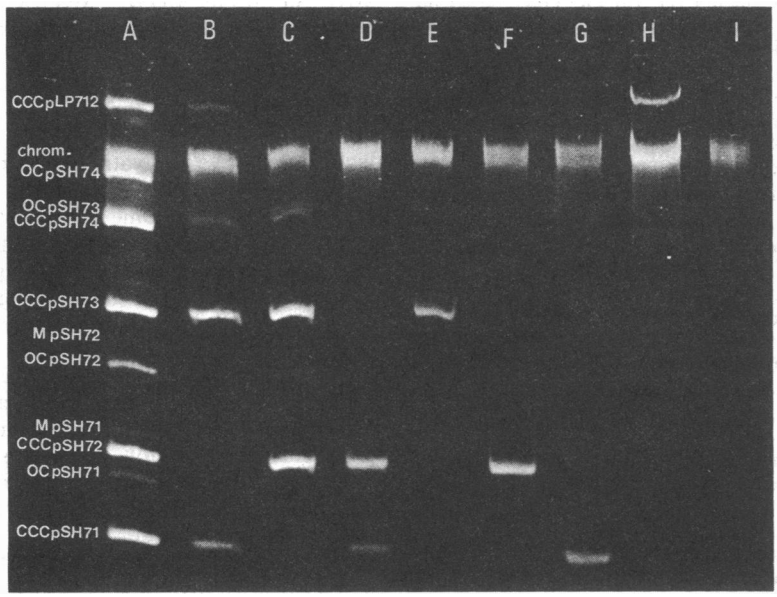

FIG. 2. Plasmid curing in *S. lactis* 712 after sequential rounds of protoplast regeneration. The parent strain SH4109 with five molecules (A) is followed by partially cured strains MG1066 (B), MG1063 (C), and MG1163 (D), which were isolated during the identification of single-plasmid-carrying derivative strains MG1261 (E), MG1362 (F), MG1365 (G), and MG1299 (H) and a plasmid-free strain, MG1363 (I). Details of the plasmid complements are shown in Tables 1 and 2, and the different molecular forms of these plasmids are indicated to the left of track A. CCC, Covalently closed circular; OC, open circular; M, multimeric.

compared with that of a plasmid-free strain and with an *S. lactis* 712 derivative carrying a complete plasmid complement. Only the 33-Md plasmid, which will subsequently be referred to as pLP712, was needed for normal growth and acid production (Fig. 3).

Deletions of pLP712. Lactose-defective derivatives of strain MG1299 were isolated by both protoplast regeneration and conventional plasmid curing treatments. Of 33 lactose-defective derivatives obtained from protoplast curing, 7 had no 33-Md plasmid, whereas 2 appeared to retain a molecule of this size. In the remaining 24 strains a plasmid smaller than 33 Md was present; the smallest molecule found was only 8 Md. In a series of acriflavine and heat curing experiments, 86% of the lactose-negative derivatives isolated had lost pLP712, whereas 14% carried a smaller molecule. Strains carrying smaller molecules were sometimes both lactose and protease negative and sometimes lactose negative but protease positive. It was also possible after protoplast regeneration to isolate lactose-positive but protease-negative derivatives that retained a plasmid smaller than 33 Md.

Restriction endonuclease digestion was used to compare these smaller molecules with plasmid pLP712. Restriction endonucleases *Bcl*I *Eco*RI, *Hae*II, and *Hin*dIII were used because these enzymes were known to produce relatively complex digestion patterns from pLP712. Digests of pLP712 were compared with those of smaller molecules present in strains with a Lac^- Prt^- phenotype (MG1422), a Lac^+ Prt^- phenotype (MG1402), and Lac^- Prt^+ phenotype (MG1523). The fragmentation patterns observed (Fig. 4) clearly showed that the smaller molecules were deleted derivatives of pLP712. In all cases the fragments produced from the smaller plasmid molecules corresponded with those from pLP712, except that one novel fragment was sometimes present; this was presumed to contain the deletion endpoints (Fig. 4). pLP712 and various smaller derivative plasmids have been analyzed by single and double digestion with restriction endonucleases chosen for the relative simplicity of the fragmentation patterns which they produce (unpublished data; Gasson et al., 94th Meet. Soc. Gen. Microbiol., abstr. no. M8). The deletion phenomenon described here was authenticated by constructing a *Bcl*I and *Bgl*II restriction and deletion map for pLP712 and the smaller plasmids shown in Fig. 4 (Fig. 5). The *Bcl*I and *Bgl*II fragment order and deletion location were determined with data from a more detailed molecular characterization of pLP712 (not shown), and a complete map of this important metabolic plasmid will be published separately. The *Bgl*II and *Bcl*I map (Fig. 5) shows the value of the deleted derivatives for locating the genetic determinants for both lactose utilization and protease production.

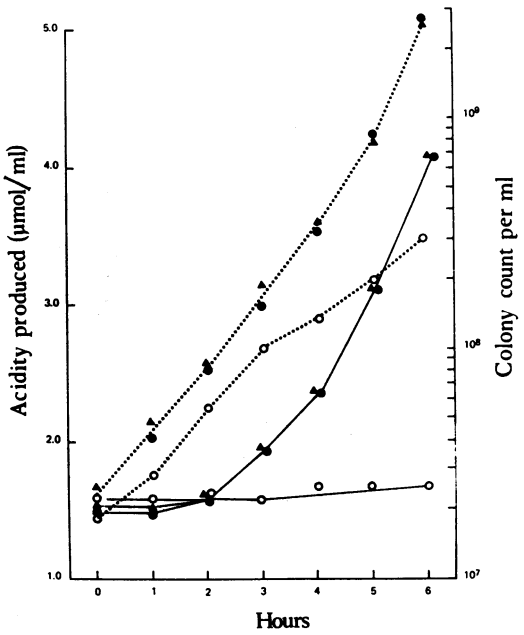

FIG. 3. Effect of *S. lactis* 712 plasmids on organism growth and acid production in milk. Growth (-----) and titratable acidity (——) of a 1% inoculum in milk are plotted against time. Data are shown for the *S. lactis* 712 derivatives SH4109 (▲), carrying a complete plasmid complement, MG1299 (●), carrying only the lactose and proteinase plasmid, and MG1363 (○), which is plasmid free.

Plasmid transfer. In *S. lactis* 712 strains carrying a full complement of cryptic plasmids, lactose and protein utilization genes have been transferred by both conjugation and transduction (1, 5, 7). These gene transfer processes were reexamined with plasmid-free recipient strains and donor strains carrying only pLP712.

In plate mating experiments (7), pLP712 was conjugally transferred from strain MG1299 to the plasmid-free, streptomycin- and rifampin-resistant derivative strain MG1614. Transfer frequency was 2×10^{-7} progeny per recipient, and the progeny colonies were of two distinct morphologies, as previously described (7). Variant aggregating progeny, denoted Lax^-, showed an elevated transfer frequency in subsequent mating experiments. In contrast to previous observations with multiple-plasmid-carrying strains (7), protease genes were always transferred together with lactose utilization genes. Normal or Lax^+ progeny carried the 33-Md pLP712 plasmid, but aggregating or Lax^- progeny contained no plasmid DNA detectable by the repeated use of standard cleared-lysate protocols. The appli-

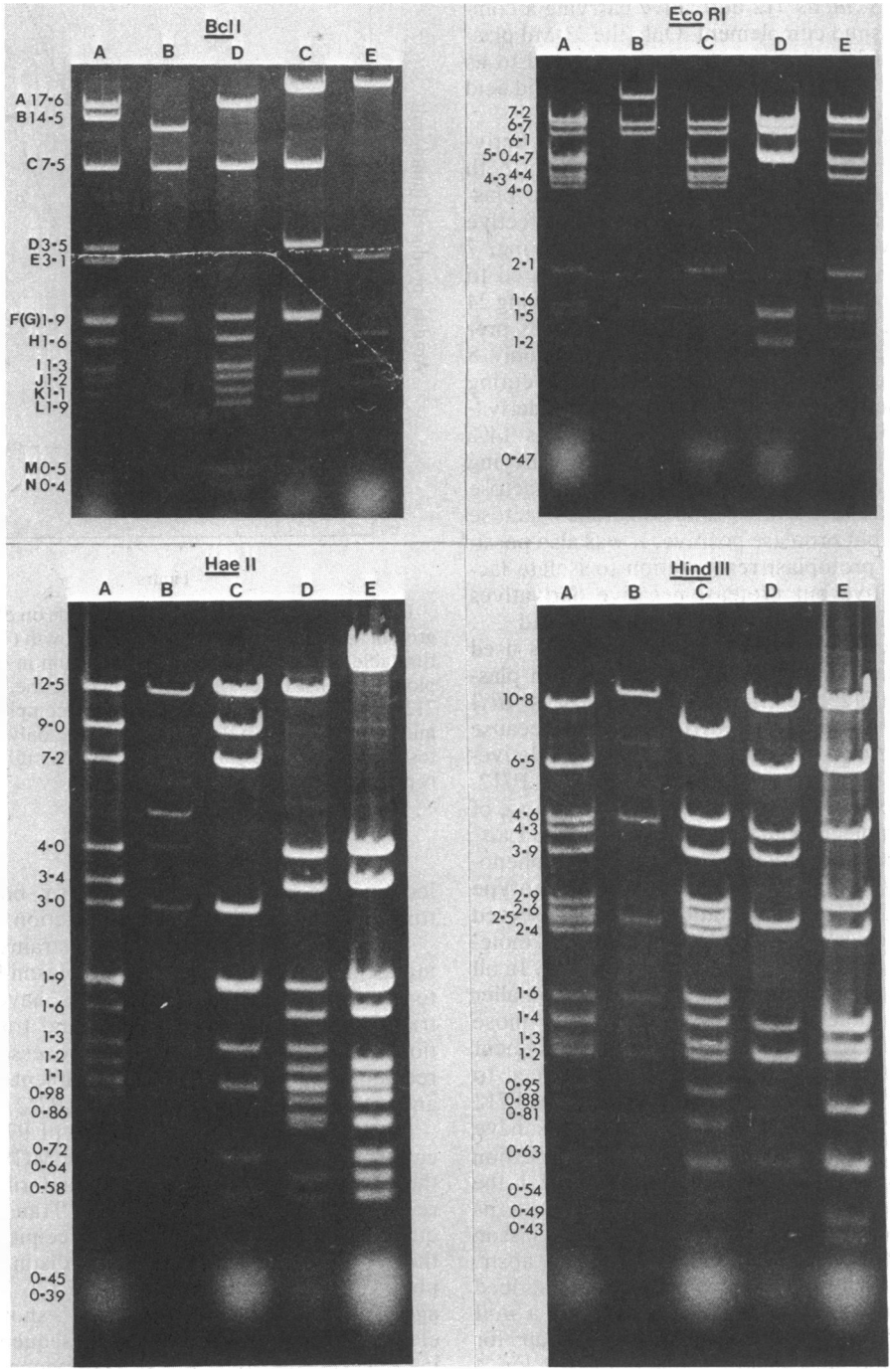

FIG. 4. Restriction endonuclease digestion of pLP712 and various deleted derivatives. Purified plasmid DNA was digested with restriction endonucleases *Hin*dIII, *Hae*II, *Eco*RI, or *Bcl*I as indicated. The plasmids used were (A) pLP712 from MG1299; (B) a Lac$^-$ Prt$^-$ deleted plasmid pMG422 from strain MG1422; (C) a Lac$^+$ Prt$^-$ deleted plasmid pMG402 from strain MG1402; (D) a Lac$^-$ Prt$^+$ deleted plasmid pMG523 from strain MG1523; and (E) a Lac$^+$ Prt$^+$ transduced plasmid pMG393 from strain MG1393. Molecular sizes (in kilobases) of DNA fragments are given for the pLP712 bands in the A tracks.

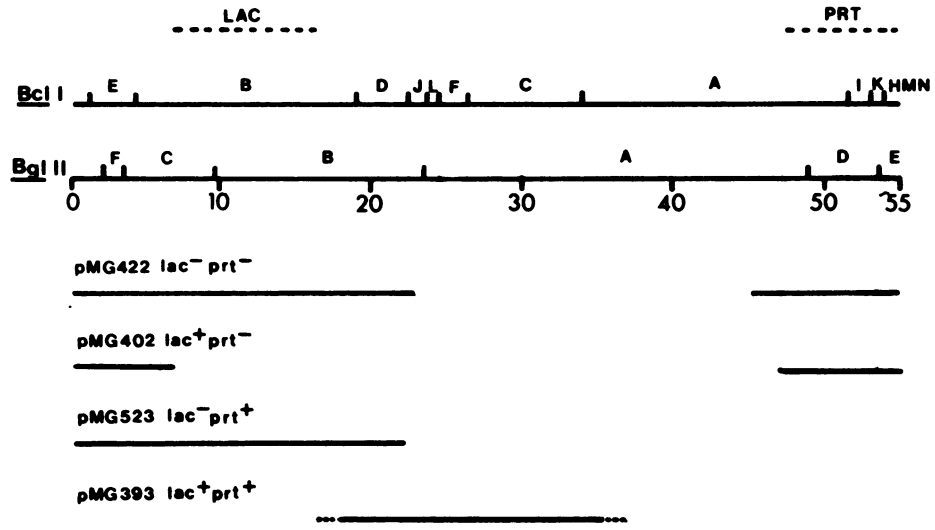

FIG. 5. pLP712 restriction endonuclease map for *BclI* and *BglII*, oriented by the single site on pLP712 for *SalI* (0 to 55 kilobases), is shown. The extent of the deletions in protoplast-induced Lac⁻ Prt⁻ (pMG422), Lac⁺ Prt⁻ (pMG402), and Lac⁻ Prt⁺ (pMG523) plasmids and in a Lac⁺ Prt⁺ transductionally shortened plasmid (pMG393) are indicated by the solid lines. The location of the Lac and Prt genes is indicated by the broken lines.

cation of a new alkaline denaturation procedure (L. Morelli and M. J. Gasson, unpublished data) to the Lax⁻ strains recently revealed the presence of larger-molecular-weight plasmids consisting of pLP712 with an insert of novel DNA (M. J. Gasson, and L. Morelli, manuscript in preparation).

Transfer of lactose genes by φT712-promoted transduction was investigated with phage induced from a φT712 lysogen of strain MG1299 and a plasmid-free recipient strain. Transduction of lactose genes occurred as previously described for multiple-plasmid-carrying strains (1, 5). Both protease-positive and protease-negative transductants were found, and in subsequent rounds of transduction the lactose gene transfer frequency was significantly increased. Transductants carried plasmids of 22 to 26 Md. These appeared, on the basis of restriction endonuclease digestion, to be deleted derivatives of pLP712 that had been selected by their accommodation inside the phage head (Fig. 4 and 5) (P. J. Warner, and M. J. Gasson, manuscript in preparation).

DISCUSSION

Plasmid curing after protoplast regeneration has already been observed in *Staphylococcus* (20), *Bacillus* (3), and *Streptomyces* (10) species. Here the phenomenon was shown for plasmid-encoded phenotypes in lactic streptococci and shown to be effective for randomly eliminating cryptic plasmid molecules. The observation that protoplast regenerants often consist of a mixture of cured and noncured cells supported conclusions from experiments with *Staphylococcus* (20) that curing occurs during the regeneration process. Previous attempts to isolate plasmid-free strains from multiple-plasmid-containing *S. lactis* strains relied on acriflavine and heat curing (unpublished data) or on the use of nitrosoguanidine (18). Sequential protoplasting and regeneration have the important advantage of being apparently nonmutagenic while providing very frequent elimination of plasmids. The plasmid-free derivative of *S. lactis* 712 obtained by this approach showed no signs of poor growth, and a strain carrying only pLP712 grew normally in its natural milk environment.

The data obtained for *S. lactis* 712 illustrate an important general application for protoplast curing. The association of an apparently plasmid-encoded phenotype with an individual molecule from a complex multiple-plasmid complement can prove to be difficult (1, 5, 7). The efficiency of plasmid curing by protoplast regeneration was sufficient to allow derivatives missing a plasmid to be isolated before changes in phenotype were sought. This inversion of the usual curing experiment provides unambiguous data for the assignment of a phenotype to a particular molecule. If a suspected plasmid phenotype remains after a particular molecule is cured, then that plasmid cannot be involved. The random loss of cryptic plasmids and plasmid bands consisting of more than one molecule would not affect this interpretation. This is clearly shown by the data for the lactose utilization phenotype of *S. lactis*

TABLE 3. Plasmid curing in *S. lactis* SH4109 and derivatives

Strain no.	Phenotype	Plasmid complement					No. of regenerants tested	No. of regenerants cured of plasmid:				
		pLP712	pSH74	pSH73	pSH72	pSH71		pLP712	pSH74	pSH73	pSH72	pSH71
SH4109	Lac^+	+	+	+	+	+	14 Lac^+	0	9	0	1	3
							16 Lac^-	4	8	1	0	2
MG1066	Lac^+	+	−	+	−	+	50 Lac^+	0		0		3
							13 Lac^-	5		0		0
MG1180	Lac^+	+	−	+	−	−	56 Lac^+	0		1		
							11 Lac^-	9		0		
MG1063	Lac^-	−	−	+	+	−	20			1	0	
MG1163	Lac^-	−	−	−	+	+	30				2	2

712 (Table 3). In lactose-defective derivatives any of the plasmids from the complement could be missing, but no consistent correlation was observed between the loss of Lac and the loss of an individual plasmid molecule. This agrees with previous results for *S. lactis* 712 obtained with conventional plasmid curing techniques (1, 7). However, if the physical loss of a plasmid molecule is related to phenotype, a clear explanation emerges. Cured derivatives missing pSH71, pSH72, pSH73, or pSH74 were isolated, in which the lactose utilization phenotype remained unchanged. Therefore, none of these plasmids controlled the Lac phenotype. In all 18 examples (Table 3) in which the 33-Md plasmid band was lost, the derivatives changed from a Lac^+ to a Lac^- phenotype, implicating plasmid pLP712 in the control of lactose utilization. The fact that Lac^- strains sometimes retained a molecule of 33 Md is not relevant to this interpretation. Such instances might be caused by the presence of an additional cryptic plasmid(s) of similar molecular weight, spontaneous Lac^- mutation, or Lac^- deletions too small to be revealed by screening on agarose gels. Clearly, protoplast curing has similar potential in other bacterial species, especially those, such as *Bacillus thuringiensis* (9), in which large plasmid complements are commonly encountered. In our laboratory this general approach has already proved useful for studying the relationship between *S. lactis* plasmid complements and nisin production (J. Hart, unpublished data).

The isolation of derivatives which carry only an individual molecule from a multiple-plasmid strain (Fig. 3) also facilitated the simple purification of that molecule for detailed study. Deleted derivatives of pLP712 were readily isolated after protoplast regeneration of a strain carrying only that plasmid. A large group of these deletions, including examples missing either or both of the lactose and protease determinants, have now been isolated. They proved to be valuable for locating these determinants on a restriction map of pLP712 (Fig. 5) (Gasson and Warner, unpublished data; Gasson et al., 94th Meet. Soc. Gen. Microbiol., abstr. no. M8). Although deletion formation was most common after protoplast regeneration, it was also encountered spontaneously and after acriflavine or heat curing. It is likely, therefore, that the pLP712 molecule is unusually prone to deletion and that this phenomenon may more widely account for the difficulty in assigning curable phenotypes to individual plasmid molecules. In *S. lactis* 712 and *S. lactis* subsp. *diacetylactis* NCDO 176 and NCDO 873, lactose-negative derivatives were found to have unchanged plasmid profiles. This might be explained by the presence of two plasmids of similar size, but might also be the result of a small deletion. Similar observations for the protease phenotype have been made in a variety of strains (1, 15, 17). These and the incomplete linkage observed between protease and lactose determinants in *S. lactis* 712 (1, 5, 7) could be explained by deletion formation. The unusually high frequency of this event in pLP712 leads to a speculative explanation for the origin of the large complement of cryptic plasmids universally found in lactic streptococci. It is conceivable, in a situation in which replication is under relaxed control, that spontaneously deleted derivatives of plasmids such as pLP712 might coexist with the parent molecule subsequently appearing to be additional, cryptic, components of the plasmid complement. Large deletions of pLP712 were not observed in *S. lactis* 712 derivatives carrying multiple-plasmid complements. In derivatives carrying pLP712 alone or one other low-molecular-weight plasmid deletions were very common. This suggests that one possible role for the cryptic plasmid complements is to stabilize a metabolically important but exceptionally unstable plasmid such as pLP712. We are currently investigating the stability and molecular relationships of the plasmids present in *S. lactis* 712 to test this hypothesis.

ACKNOWLEDGMENTS

I thank Jan Elkins for invaluable technical assistance and Phil Warner, Joe Hart, and Lyndon Davies for discussion of the work.

LITERATURE CITED

1. **Davies, F. L., and M. J. Gasson.** 1981. Review of the progress of dairy science: genetics of lactic acid bacteria. J. Dairy Res. **48**:363–376.
2. **Davies, F. L., H. M. Underwood, and M. J. Gasson.** 1981. The value of plasmid profiles for strain identification in lactic streptococci and the relationship between *Streptococcus lactis* 712, ML3 and C2. J. Appl. Bacteriol. **51**:325–337.
3. **Fowler Edger, N., K. Orzech McDonald, and W. F. Burke.** 1981. Plasmid curing in *Bacillus subtilis*. FEMS Microbiol. Lett. **12**:131–133.
4. **Gasson, M. J.** 1980. Production, regeneration and fusion of protoplasts in lactic streptococci. FEMS Microbiol. Lett. **9**:99–102.
5. **Gasson, M. J.** 1982. Identification of the lactose plasmid in *Streptococcus lactis* 712, p. 217–220. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
6. **Gasson, M. J., and F. L. Davies.** 1980. Conjugal transfer of the drug resistance plasmid pAMβ in the lactic streptococci. FEMS Microbiol. Lett. **7**:51–53.
7. **Gasson, M. J, and F. L. Davies.** 1980. High-frequency conjugation associated with *Streptococcus lactis* donor cell aggregation. J. Bacteriol. **143**:1200–1264.
8. **Gasson, M. J., and F. L. Davies.** 1980. Prophage-cured derivatives of *Streptococcus lactis* and *Streptococcus cremoris*. Appl. Environ. Microbiol. **40**:964–966.
9. **Gonzalez, J. M., and B. C. Carlton.** 1980. Patterns of plasmid DNA in crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*. Plasmid **3**:92–98.
10. **Hopwood, D. A.** 1981. Genetic studies with bacterial protoplast. Annu. Rev. Microbiol. **35**:237–272.
11. **Kempler, G. M., and L. L. McKay.** 1979. Characterization of plasmid deoxyribonucleic acid in *Streptococcus lactis* subsp. *diacetylactis*: evidence for plasmid-linked citrate utilization. Appl. Environ. Microbiol. **37**:316–323.
12. **Kempler, G. M., and L. L. McKay.** 1979. Genetic evidence for plasmid-linked lactose metabolism in *Streptococcus lactis* subsp. *diacetylactis*. Appl. Environ. Microbiol. **37**:1041–1043.
13. **Kempler, G. M., and L. L. McKay.** 1980. Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl. Environ. Microbiol. **39**:926–927.
14. **Kempler, G. M., and L. L. McKay.** 1981. Biochemistry and genetics of citrate utilization in *Streptococcus lactis* subsp. *diacetylactis*. J. Dairy Sci. **64**:1527–1539.
15. **Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin.** 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. Appl. Environ. Microbiol. **35**:592–600.
16. **Kuhl, S. S., L. D. Larsen, and L. L. McKay.** 1979. Plasmid profiles of lactose-negative and proteinase-deficient mutants of *Streptococcus lactis* C10, ML3, and M18. Appl. Environ. Microbiol. **37**:1193–1195.
17. **McKay, L. L., and K. A. Baldwin.** 1975. Plasmid distribution and evidence for a proteinase plasmid in *Streptococcus lactis* C2. Appl. Microbiol. **29**:546–548.
18. **McKay, L. L., K. A. Baldwin, and J. D. Efstathiou.** 1976. Transductional evidence for plasmid linkage of lactose metabolism in *Streptococcus lactis* C2. Appl. Environ. Microbiol. **32**:45–52.
19. **McKay, L. L., K. A. Baldwin, and E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. **23**:1090–1096.
20. **Novick, R., C. Sanchez-Rivas, A. Gruss, and I. Edelman.** 1980. Involvement of the cell envelope in plasmid maintenance: plasmid curing during the regeneration of protoplasts. Plasmid **3**:348–358.
21. **Pechmann, H., and M. Teuber.** 1980. Plasmid pattern of group N (lactic) streptococci. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **101**:133–136.
22. **Stadhouders, T.** 1961. The hydrolysis of protein during the ripening of cheese: some methods to accelerate the ripening. Neth. Milk Dairy J. **15**:161–164.